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Optimized Surface-enhanced Raman Scattering (SERS) Colloids for the Characterization of Microorganisms

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We report on a comparison of silver colloid preparation methods for the *in situ* analysis of microorganisms based on surface-enhanced Raman scattering (SERS). Different colloid sols were tested and optimized regarding their suitability as SERS substrates. The silver sols produced by a modified procedure of Leopold and Lendl gave an enhancement factor of the Raman signal in the order 10^8 for the test molecule, crystal violet. Furthermore, this SERS substrate was successfully applied for the *in situ* detection and identification of microorganisms immobilized on an immunoassay. The colloid preparation was carried out at room temperature and the colloids were stable for weeks. This silver nanoparticle preparation method for the label-free *in situ* detection of microorganisms successfully combines SERS with immunoassays. Hence, it has a great potential for the high-throughput detection of biomolecules.

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Introduction

Since the first observation of the surface-enhanced Raman scattering (SERS) effect, a variety of metals (Ag, Au and Cu) have been applied for its generation. These metals can be utilized in different formats, *e.g.* as metal plates, colloids, rods, and coatings on surfaces *etc.*[1-5] The SERS effect enhances low Raman signals, and allows obtaining fingerprint SERS spectra of different biological systems, which are not accessible by normal Raman spectroscopy due to its limited sensitivity[6] (for recent review see Ref. 7). A particular advantage of SERS for the characterization of biomolecules is the fact that it can be carried out in an aqueous medium, since water is a weak Raman scatterer. There has been huge progress in characterizing and identifying microorganisms with colloidal SERS substrates in the past.[8-11] A large variety of molecules and larger molecular structures can be analyzed with high precision. Enhancement occurs in close proximity of metallic nanostructures either by electromagnetic ("localized surface plasmon resonance") and/or chemical ("charge transfer") effects. Moreover, closely spaced interacting particles, the so-called "hot spots", seem to provide extra field amplification, resulting in enhancement factors of up to 10^{14} – 10^{15}, allowing even for single-molecule detection.[12-15] Such high amplification can only be obtained at very restricted areas, and is hardly reproducible. Highly SERS-active inter-particle spaces can be produced by controlled agglomeration of a colloid.[15] Adding salt to the colloid sol causes the metal particles to coagulate in the dependence of the salt concentration. This effect is called the chloride effect described by Li *et al.*[16] In order to obtain reproducible enhancement factors and spectral features by SERS measurements, the reproducible production of SERS substrates featuring uniform shapes and sizes of nanostructures is crucial. This is an essential prerequisite to achieve stable surface plasmon resonance frequencies, which again result in reproducible SERS band positions and band intensities. Another important contribution to reproducible SERS measurements is the controlled aggregation of metal nanoparticles. To produce high Raman enhancements, the nanoparticles must be within a certain distance to the analyte and have a size of 20 – 100 nm. If the colloid sol is polydisperse, this can limit the reproducibility of enhancements. For the synthesis of a colloid sol as a substrate for sensitive and reproducible SERS measurements, different parameters have to be taken into consideration *e.g.* the reaction temperature, stirring time and power, reactant's volume, as well as the way and order of the reactant's addition.

Silver colloids were first presented as SERS substrates by Creighton *et al.* in 1979.[17] They proved that superior enhancement can be achieved using silver surfaces in comparison to gold structures. Ooka *et al.*[18] showed that silver colloids interact with both amine and carboxylate groups. The lone-pair electrons of the silver atom interact with the respective empty orbital of the functional groups. A silver surface in contact with water is slightly electropositive; hence, it shows an affinity to electronegative regions on bacteria cell walls due to electrostatic interactions, in addition to van-der-Waals forces.

In this paper, several colloid sols were tested as SERS substrates for the analysis of bacteria. We describe the effect of changing the reaction parameters on the nanoparticles and on the resulting Raman cross sections. We have modified a Ag colloid preparation procedure, originally developed by Leopold and Lendl,[19] for implementation as SERS substrates on microorganism detection. Using this colloid sol, enhancement factors of over 10^8 were reached with the test molecule, crystal violet (CV). CV is a widely used Raman reporter molecule that is also implemented as a labelling molecule for microorganism SERS detection.[20] Kneipp *et al.* showed that CV provides an extremely large effective cross section, and carried out single

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molecule analysis in the presence of Ag colloid sol.[12] This dye has an extreme of the increase cross section by SERS and is suitable as a test molecule.

The advantages of the proposed procedure are the straightforward, fast and inexpensive production and high reproducibility as well as a long shelf life of the resulting particles. We have optimized the rate of silver particle agglomeration as well as the distribution of the particles on the cell-wall, resulting in the formation of "hot spots" on the bacteria surfaces. As a result, we were able to detect two different microorganisms label-free, on an immunoassay platform in water, with high resolution.

Experimental

Reagents and chemicals

Methanol, ethanol, sulfuric acid, hydrochloric acid, DMF, triethylamine, di(*N*-succinimidyl)-carbonate (DSC), dimethylaminopyridine (DMAP), Pluronic F 127, trehalose, 3-glycidoxypropyltrimethoxysilane (GOPTS), NaOH, NaN_3, Na_2CO_3, $NaHCO_3$, NaCl, casein, $NH_2OH{\cdot}HCl$, KOH, $AgNO_3$, $NaBH_4$ and trisodium citrate were bought from Sigma Aldrich (Taufkirchen, Germany) and used without further purification. Glass slides ($26 \times 76 \times 1$ mm) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Microtiter plates 384-well (BD FalconTM, reference 353265, natural polypropylene, flat bottom, non-sterile, LOT 06291155, low binding) were purchased from VWR International GmbH (Darmstadt, Germany). Hellmanex II was purchased from Hellma (Müllheim, Germany). Diamino-polyethylene glycol 2000 Da (Jeff-Amine) was a gift from Huntsman Holland (Rozenburg, Netherlands).

Anti-*Legionella* antibodies (pAB rabbit, Cat #B65051G) and 2 – 2.5 g/L anti-*Salmonella* antibodies (pAB goat, Cat #G5V61-500) were both bought from BioDesign International (Saco, USA); autoclaved *Legionella pneumophila* cells were delivered from Max-Pettenkofer Institut (Munich, Germany), and heat-inactivated *Salmonella typhimurium* ATCC 14028 cells were provided from Instituto Zooprofilattico Sperimentale DellÀbruzzo e del Molise "G. Caporale" (Teramo, Italy).

Phosphate buffered saline (PBS) contained 145 mM NaCl, 70 mM K_2HPO_4 and 10 mM KH_2PO_4, and was adjusted to pH 7.6 and filtrated before use. Carbonate buffer contained 35 mM $NaHCO_3$, 15 mM Na_2CO_3 and 3.1 mM NaN_3, and was adjusted to pH 9.6 before use. Milli-Q water grade was used for all solutions.

Colloid preparations

For all described colloid preparation procedures, a glassware washing procedure, including a KOH-bath, followed by an HCl-bath, was carried out. Residual particles on glass walls can act as catalysts, initiating premature agglomeration.

Silver colloid sol A was prepared using a procedure of Creighton.[17] Briefly, 14.98 mg of $NaBH_4$ (0.4 mmol) was dissolved in 60 mL of Milli-Q water in excess in an ice bath and split in 6 mL batches; 0.85 mg $AgNO_3$ (0.005 mmol) in 2 mL of Milli-Q water were added drop-wise under vigorous stirring (1200 rpm) to generate particles with a narrow size distribution. The resulting lightly greyish sols were stored in the dark at 4°C.

Silver colloid sol B was prepared using a procedure according to Lee and Meisel.[21] Briefly, 18 mg (0.1 mmol) of $AgNO_3$ were dissolved in 100 mL Milli-Q water in a 250-mL round bottom flask and divided in batches of 25 mL. These batches were heated to the solution's boiling point, and 0.2 mL (0.07 mmol) of 1% trisodium citrate were quickly added. The mixture was left stirring (600 rpm) for 1 h under reflux. Finally, the heat source was removed and the solution was stirred (600 rpm) for another 30 min. After reaching room temperature, each silver colloid sol B batch was tested for its particle size distribution using a UV-Vis spectrometer (UV-DU 650, Beckmann Coulter, CA). The greyish colloid sols were stored in the dark at 4°C.

Silver colloid sol C was prepared using a modified procedure of Leopold and Lendl.[19] Briefly, 17 mg (0.1 mmol) of $AgNO_3$ was dissolved in Milli-Q water (10 mL). Then, 100 mL of a 11.6 mg (0.17 mmol) $NH_2OH{\cdot}HCl$ solution containing 3.3 mL of NaOH (0.1 M) was prepared and divided in 9 mL batches in centrifuge tubes. To the reducing agent, 1 mL of $AgNO_3$ was added at a flow rate of 0.67 mL s^{-1} without stirring. Finally, the centrifuge tube was inverted once to complete the mixing. The particle size distribution of each silver colloid solution was tested using a UV-Vis spectrometer (Beckmann UV-DU 650, Beckmann Coulter, CA) (the procedure is described later). The yellow/greenish colloid sols were stored in the dark at 4°C. TEM pictures were developed with JEM 2010 (LaB_6 cathode; JEOL, Munich, Germany) with an accelerating voltage of 200 kV.

Microarray platform

The bacteria were resuspended in carbonate buffer containing 0.02% NaN_3 and incubated for 1 h on a spotted glass chip containing the respective antibodies. In order to remove unspecifically bound microorganisms, the chip was thoroughly washed in PBS. The chip was then placed into a polycarbonate tray that was filled with silver colloid sol C containing 0.03 M NaCl.

SERS measurements

All SERS spectra were obtained with a Renishaw 2000 Raman microscope system (Renishaw, UK), using a He-Ne laser (λ_0 = 633 nm; laser power at spot, 7 mW). Wavelength calibrations were performed by measuring silicon wafers through a ×50 objective, evaluating the first-order phonon band of Si at 520 cm^{-1}. The SERS measurements on CV were carried out in a microtiter plate using a ×50 objective with a numerical aperture (NA) of 0.75. In a cavity of the plate, CV was added to a diluted silver colloid sol (1:6) containing the desired salt concentration (0.01 – 0.04 M). The colloid sol was diluted, so that no excessive agglomeration of colloids would hinder the SERS enhancement. If not stated differently, the exposure time was set to 1 s for all CV spectra presented in this study. The signal-to-noise ratios (SNR) of spectra collected from colloid sols A, B, and C were compared. Mean values and standard deviations of three measurements were calculated. The limit of detection was defined as SNR ≥ 3 in this experiment (Table 1). SERS spectra on bacteria were carried out using a water immersion objective (×63, NA 0.9, lateral resolution ~1 μm) with an exposure time of 10 s.

Results and Discussion

Characterization of the silver colloids

According to Petit *et al.*,[22] the size distribution of colloidal nanoparticles can be derived from extinction spectra obtained by an UV-Vis spectrometer. A narrow absorption peak with steep flanks indicates monodispersed particles. In such a case, the diameter, *D*, in nanometers of the silver nanoparticle can be calculated using

Table 1 SNR table of SERS enhancements

Reducing agent	NaCl/M	CV/M							
		10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Hydroxylamine hydrochloride	0.00	17 ± 2	30 ± 5	7 ± 0.2	<3	<3	<3	<3	<3
	0.01	47 ± 7	59 ± 6	19 ± 1	5 ± 0.3	<3	<3	<3	<3
	0.02	37 ± 10	65 ± 7	21 ± 5	<3	<3	4 ± 0	5 ± 0.5	4 ± 0.5
	0.03	33 ± 7	212 ± 16	26 ± 3	17 ± 2	36 ± 7	6 ± 0.8	7 ± 0.5	9 ± 4
	0.04	39 ± 3	49 ± 8	58 ± 33	4 ± 0.2	<3	11 ± 4	9 ± 1	6 ± 1
Trisodium citrate	0.00	97 ± 7	14 ± 4	5 ± 1	<3	<3	<3	<3	<3
	0.01	79 ± 17	104 ± 8	26 ± 5	26 ± 13	3 ± 0.5	20 ± 20	23 ± 10	13 ± 12
	0.02	88 ± 23	98 ± 33	61 ± 15	18 ± 11	13 ± 2.8	24 ± 17	7 ± 1	18 ± 12
	0.03	117 ± 18	124 ± 10	98 ± 12	39 ± 14	22 ± 12	6 ± 2	23 ± 8	24 ± 2
	0.04	111 ± 8	119 ± 8	118 ± 14	32 ± 5	4 ± 0.5	17 ± 13	18 ± 7	14 ± 0.5
Sodium borohydrate	0.00	91 ± 1	80 ± 5	13 ± 0.5	4 ± 2				
	0.01	30 ± 5	15 ± 2	8 ± 1	<3				
	0.02	66 ± 12	38 ± 6	6 ± 2	4 ± 0.5				
	0.03	75 ± 12	68 ± 4	7 ± 2	<3			<3	
	0.04	68 ± 11	84 ± 13	13 ± 3	4 ± 0.5				
Normal Raman	0.00	<3	<3	<3	<3	<3	<3	<3	<3

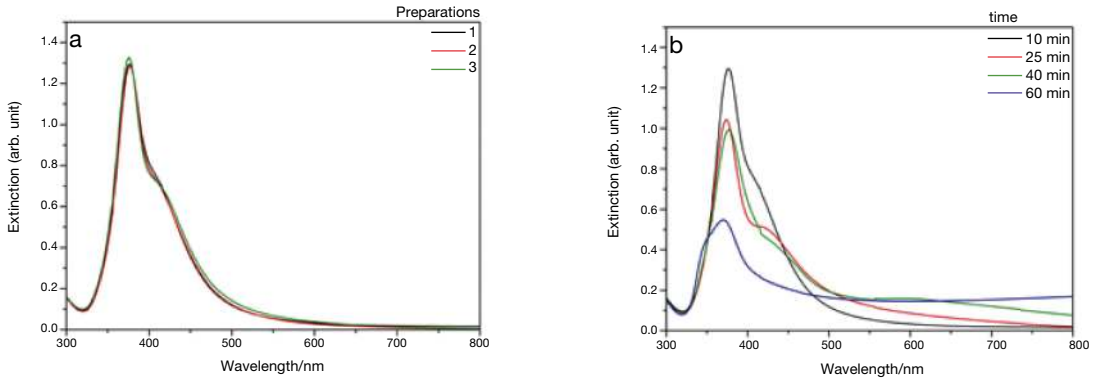

Fig. 1 UV-Vis spectra of silver colloid sol A. (a) The synthesis was reproducible over three preparations. (b) Sol A showed limited stability. After 25 min a shoulder at 430 nm developed significantly.

$$\mathrm{FWHM} = 50 + \frac{230}{D}. \quad (1)$$

A red-shift of the absorbance maximum can be caused by two phenomena: a wide distribution of particle sizes and/or larger particles, or by agglomeration of the primary particles. Shoulders in the absorption spectra appear when the particles are polydisperse. The particle sizes correlate to the absorption signal intensity, *i.e.* stronger absorption indicates smaller particles.

Reproducibility of the colloid preparation

Colloid sol A. By dividing the original reactant volumes into ten batches, where the tenth batch was not used due to a high level of uncertainty, a more uniform development of silver colloids was achieved. The plasmon absorption maximum lies at 376 nm for three separate preparations, which indicates good reproducibility (Fig. 1a). Significant changes of the absorption spectra, such as absorption band broadening (Fig. 1b), indicate very limited stability of the colloids, making them unsuitable for routine SERS measurements. As a consequence, Eq. (1) is not valid for these colloids.

Colloid sol B. The original reactant volumes were divided in ten batches, where the tenth batch was not used due to a high level of uncertainty. However, the plasmon absorption maximum lied between 417 and 441 nm for five repeated preparations, indicating a limited reproducibility in their production (Fig. 2a). Additionally, a widening of the extinction signature in the UV at 350 nm suggests that the sol is polydispersed. Thus, Eq. (1) cannot be applied to calculate the particle diameter. These colloids had a shelf life of over three weeks (Fig. 2b), but the limited reproducibility of the production procedure restrains the applicability as SERS substrates.

Colloid sol C. Again, the stock solutions were divided into ten batches. An important aspect of the silver sol preparation was the rate of silver nitrate addition. It was found that a rapid addition using an Eppendorf pipette is the optimal procedure. Silver nitrate was added while swirling the pipette once over the reducing agent, which maintained stagnant. After rapid addition, the mixture was carefully swirled by rotating the centrifuge tube once. The final monodispersed colloidal solution had a yellow/greenish color, and contained particles of ~26 nm (Fig. 3a). The absorption maximum was found at 403 nm for all analyzed batches, showing good reproducibility throughout

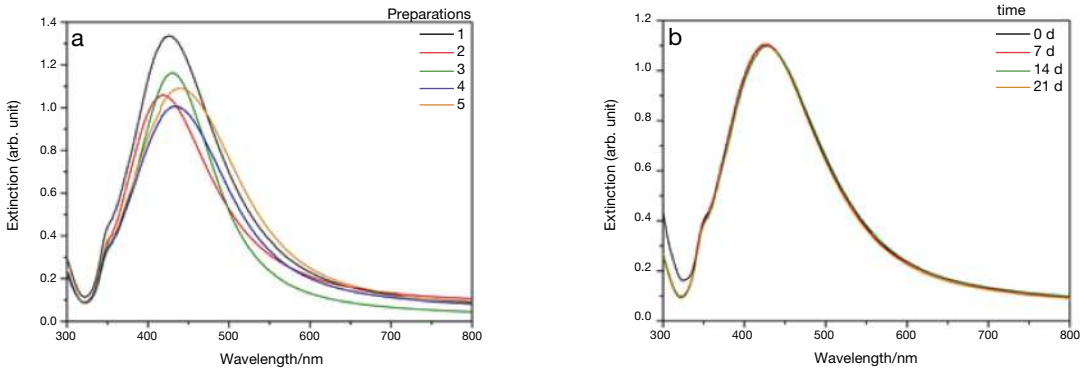

Fig. 2 UV-Vis spectra of silver colloid sol B. (a) Limited reproducibility was achieved throughout five preparations. (b) Sol B was stable for more than three weeks.

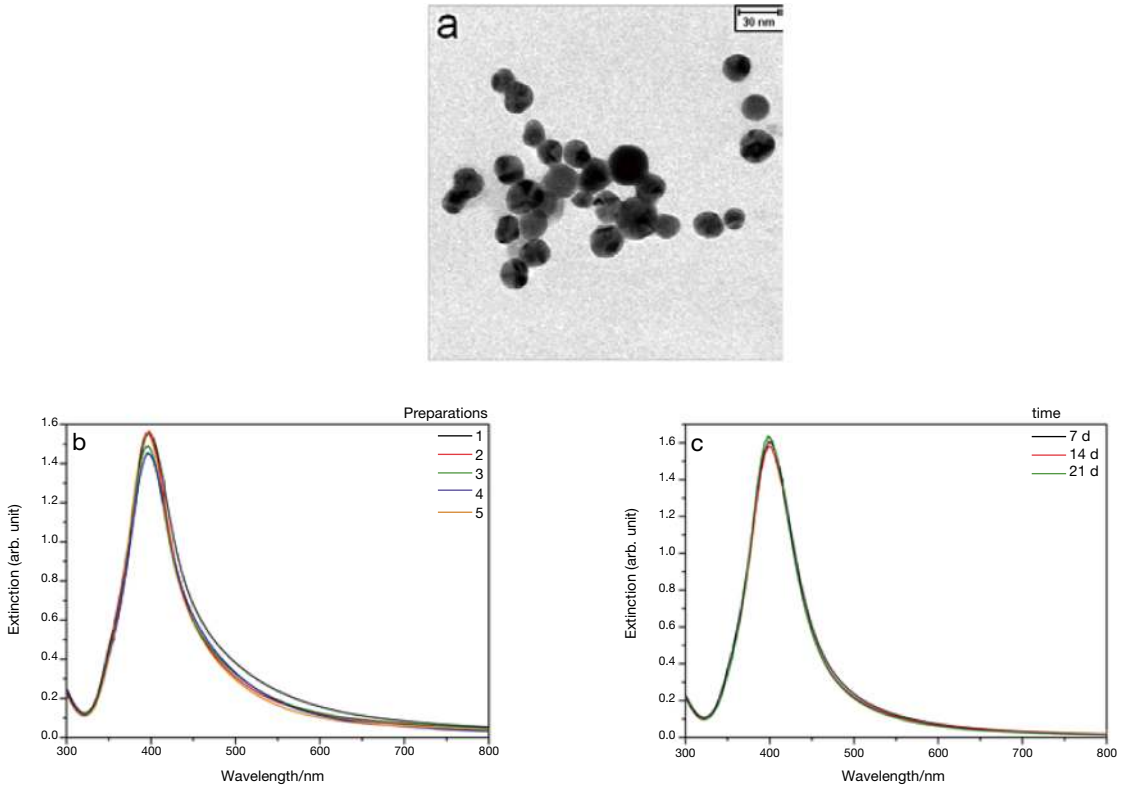

Fig. 3 Characterization of silver colloid sol C. (a) TEM image shows the narrow size distribution of the colloid sol with particle sizes of ~26 nm. (b) UV-Vis spectra illustrate a reproducible production over five preparations. (c) UV-Vis spectra of colloid sol C show substance stability of over three weeks.

Table 2 Colloidal sol parameters

Colloid sol	λ_{max}/nm	Particle diameter (D)/nm	Stability	Enhancement factor	Reaction temperature/°C	Reproducibility of production
A	376	n.a.	<25 min	10^4	10	Good
B	417 - 441	n.a.	>3 weeks	10^8	100	Moderate
C	403	26	>3 weeks	10^8	19	Good

n.a., Not applicable.

five separate preparations (Fig. 3b). These colloids had a shelf life of over three weeks, an important feature regarding their routine applications as SERS substrates (Fig. 3c).

Enhancement factor

Colloids A, B and C were tested on CV regarding their enhancement and stability of the Raman signal. Sodium

chloride in concentrations between 0.01 and 0.04 M was used as an agglomerating agent and added to the different colloid solutions, containing various CV concentrations (10^{-5} – 10^{-12} M), shortly before single Raman measurements. After adding chloride to metal colloids, their stability declined with time. Due to this fact, the salt was added to the colloids shortly before the colloids were used to obtain accurate and comparable results. We were able to detect pure CV down to 10^{-4} M using normal Raman (NR) (not shown). Here, neither salt nor colloids were added.

In the case of the colloid sol produced by sodium borohydride (colloid sol A), factors of 10^4 were obtained without chloride, and using NaCl concentrations of 0.02 and 0.04 M.

Colloids produced with tri-sodium citrate (colloid sol B) reached an enhancement of factor 10^8. This increase was obtained by adding 0.01 to 0.04 M of NaCl. When no salt was added, a signal enhancement of factor 10^3 of CV Raman scattering was obtained using these colloids. By SERS measurements the addition of 0.03 M NaCl was found to give the largest signal enhancement of CV using colloid sol C. In this case, we obtained a signal amplification factor of 10^8. If no salt was added to these colloids, a signal intensification factor of 10^2 was detected. In all further experiments involving microorganisms, the silver colloids C prepared after the modified procedure of Leopold and Lendl were used and 0.03 M NaCl were added before mixing to the analytes. Colloid sol B was not employed due to its limited reproducibility. For a summary of the results see Table 2.

We tested the SERS measurements employing an Ar^+ laser (514 nm). The results proved our hypotheses that due to salt addition, a red-shift of the plasmon resonance occurred. Hence, the use of a smaller laser wavelength resulted in a lower SERS enhancement.

SERS on bacteria

Bacteria (*Legionella pneumophila* and *Salmonella typhimurium*) were immobilized onto an amino-polyethylene glycol-coated surface containing anti-*Legionella* and anti-*Salmonella* antibodies, respectively. Colloid sol C was added to the platform prior to SERS measurements. No significant background signals were revealed from the surface material, although a certain level of fluorescence was observed. The extent and the procedure of Ag colloid agglomeration are important for the success of the SERS measurements. Sodium azide was added to the bacteria to obtain a directed formation of particle agglomerates onto the bacterial cell-wall.[6] The time of the measurement was 10 s for each spectrum. Using silver colloids C with 0.03 M NaCl, which gave the highest enhancement factor of the test molecule CV, both *Legionella pneumophila* and *Salmonella typhimurium* revealed specific SERS spectra (see Figs. 4 and 5). In a comparison with NR spectra of *L. pneumophila* and *S. typhimurium* (not shown), we have obtained high enhancement factors using the colloids produced by the modified procedure of Leopold and Lendl. The vibrational modes of the analytes are listed in Table 3. Both, the COO^- and NH_2 vibrational modes are present in the fingerprint spectra of *Legionella* and *Salmonella* (Table 3). The presence of these modes suggests that at least some of the adsorbed silver colloids interact with the analyte through amine and carboxylate groups. The bands in the region 1250 – 1310 cm^{-1} are assigned to Amide III, which primarily involves NH bending and ν(CN).[23,24] The bands at 1540 – 1645 cm^{-1} are assigned to Amide II, which arises mostly from NH bending.[9] The bands at 1640 – 1680 cm^{-1} are assigned to Amide I, which involves predominantly ν(CO) and ν(CN).[23]

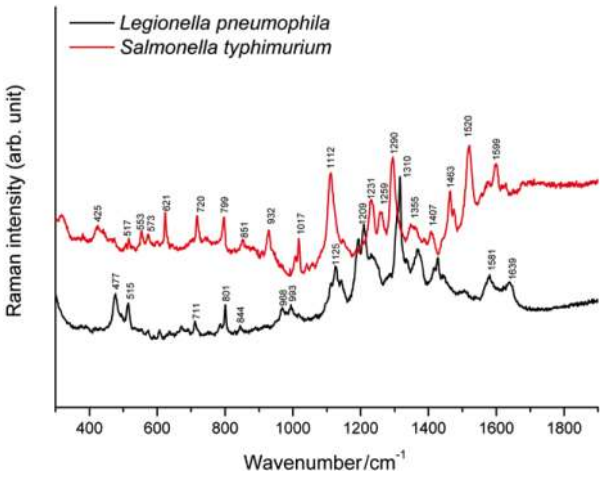

Fig. 4 SERS spectra of *S. typhimurium* (red) and *L. pneumophila* (black).

Conclusions

We studied the suitability of different Ag colloid sols as SERS substrates for the label-free *in situ* analysis of microorganisms. Comparing several preparation procedures and optimizing

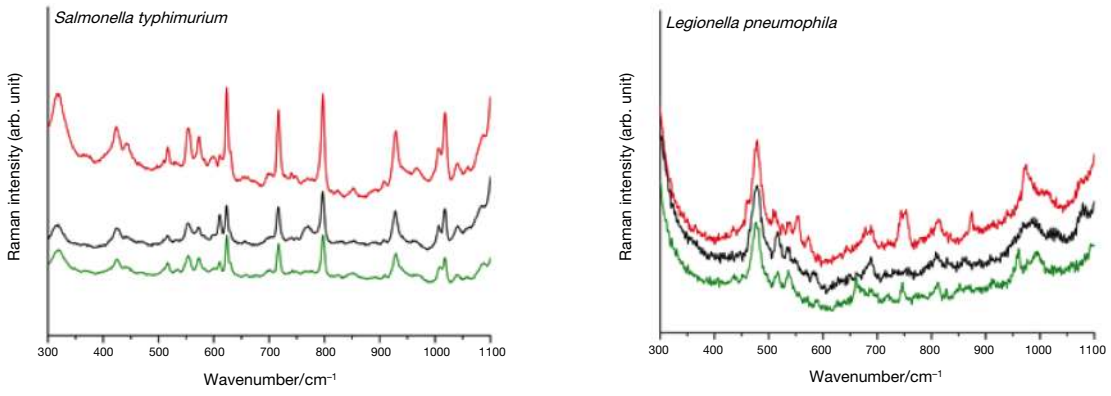

Fig. 5 Fingerprint spectra collected from *S. typhimurium* and *L. pneumophila*.

Table 3 SERS vibrational modes

Vibration mode	Wavenumber/cm^{-1}	*L. pneumophila*	*S. typhimurium*
Amide I	1640 - 1680 [23]	s	w
Amide II	1540 - 1645 [9,20,25]	s	s
$\delta(CH_2)$ saturated lipids	1440 - 1460 [23]	w	s
$\nu(COO^-)$ symmetric	1360 - 1440 [23]	s	s
Amide III	1250 - 1310 [9,23-25]	s	s
(CC) ring breath. assym.	1150 - 1160 [23]	w	w
(CO) ring breath. assym.	1150 - 1160 [23]	w	w
ν(CC) aromatic ring (Phe)	~1000 [9]	w	s
$\rho(CH_2)$	720 - 730 [9]	w	s
Phe skeletal	625 [9]	w	s

δ, Deformation; ν, stretching; ρ, rocking; Phe, phenylalanine; breath., breathing; s, strong; w, weak.

different parameters, an optimal approach was identified. We prepared stable, monodisperse silver colloid sols in a reproducible way by reducing silver nitrate with hydroxylamine hydrochloride, according to a modified procedure of Leopold and Lendl. These colloids showed a long shelf life and their implementation for SERS measurements was successful. We investigated the effect of the agglomeration rates in the increase of the SERS cross section of CV. The optimal agglomeration of the particles obtained with the test molecule CV was successfully implemented in the reproducible detection of two different microorganisms.

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